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13. ABSTRACT *Maximum 200 words)*

Malaria chemotherapeutic studies in mice included 6 different testing systems. **1)** A primary antimalarial blood schizonticidal test (MM) where in 10 years 20,500 compounds were evaluated against *Plasmodium berghei* with 2,100 exhibiting antimalarial activity. **2)** A casual prophylactic test (RP) to detect compounds active against the tissue schizonts where in 5 years over 600 compounds were tested with 380 active. **3)** A secondary antimalarial test (Ag) were in 10 years over 2000 compounds were evaluated in a variety of different protocols. Compounds were tested for cross resistance patterns against a battery of drug-resistant parasites. New lines resistant to mefloquine and halofantrine were developed. In a comparative study it took longer to induce resistance to the R-stereoisomer than the S-stereoisomer of a floxacrine racemate analog. In a modified multidose MM test artelinic acid is the most active analog of artemisin. When the fatty acids in the membranes

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of parasites and host red blood cells are changed to an increased level of polyunsaturated (Pufa) omega-3 (n-3) fatty acids in mice with reduced levels of vitamin E the antimalarial activity of qinghaosu can be potentiated. Even, without drug intervention mice can be cured when maintained on vitamin E-deficient diet high in Pufa n-3 fatty acids. *Plasmodium vinckei* parasites, unlike *P. berghei* and *P. yoelii*, are not sensitive to this change in fatty acids and vitamin E levels. **4)** In a primary screen for African trypanosomes (RR) 16,688 compounds were tested with 784 exhibiting activity. **5)** In a drug-resistant African trypanosome test 261 compounds were tested against lines resistant to either melarsoprol, suramin or pentamidine. **6)** In a Chagas' disease test 700 compounds were tested with 50 exhibiting activity.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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__✓__In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Carla L Agee Jr Date 9/27/90

TABLE OF CONTENTS

INTRODUCTION

Malaria has been the most important infectious disease the world has ever known. Currently over 400 million people are estimated to be infected with malaria leading to 2.5 million deaths. Controlling malaria has been a major problem because of three factors, ineffective drugs, ineffective vector control and no malarial vaccine. Drug-resistant parasites have emerged to *Plasmodium falciparum* and *Plasmodium vivax* the two most important species of human malaria. Chloroquine has been able to control *P. vivax* infections but not *P. falciparum* in the past 10 years. The remaining drug armlementation has been drastically reduced because of either toxicity or cross resistance with chloroquine. Amodiaquine has been retired because of the occurrence of agranulocytosis reported in some treated patients. Parasites previously sensitive to quinine have now often become cross resistant with chloroquine and consequently not effective against chloroquine-resistant *P. falciparum*. Severe toxicity problems (Stevens Johnson syndrome) have emerged in some patients taking a combination of sulfadoxine and pyrimethamine (Fansidar®). Because of this life threatening toxicity from Fansidar® coupled with emergence of *P. falciparum* resistant parasites the use of this drug combination is no longer advocated. Mefloquine, the first antimalarial agent emerging from this U.S. Army sponsored program, has been effective in many areas of the world, however, drug resistant *P. falciparum* parasites have emerged in many areas of the world and some toxicity problems have also emerged. The second drug emerging from this same program was halofantrine. It too has proven effective against chloroquine-resistant *P. falciparum* but again some resistant parasites have been reported recently. Novel approaches to reverse chloroquine resistance have been reported in *in vivo* systems and in murine and primate models but this has not been studied in humans because of anticipated toxicity problems. One new series of compounds emerging from the original chinese antimalarial compound **"Qinghaosu"** have shown good activity with lower than desired curative effects in humans. These compounds are very fast acting not long lasting, and with recrudescence rates approach 10-20% in certain geographic areas. They do not look like they will be the drug to replace chloroquine. Therefore new chemicals active against malaria and especially chloroquine-resistant malaria need to be identified. The two new drugs, mefloquine and halofantrine will help in this battle but they are not going to win the war. New compounds are needed to fill in and take over when these fail. We have established a drug

testing system (MM test) to identify new compounds against the asexual blood stages of malaria which has proved to be reliable and is able to identify all previously know antimalarial agents. This test is also useful in identifying the most active analogs of a compound emerging from a lead directed drug synthesis program.

New compounds active against the exoerythrocytic stages of malaria (sporozoite, exoerythrocytic schizonts and hypnozoites) are needed to replace the toxic primaquine. Samples of nine different chemical classes of compounds have exhibited activity against the liver stages of malaria, but primaquine remains the only one used today. It can not be prescribed as a prophylactic compound because of toxicity problems. It has a poor therapeutic index and has been shown to cause hemolytic anemia in persons with a deficiency in the enzyme glucose 6-phosphate dehydrogenase.

We have developed a test (RP test) in mice using *Plasmodium yoelii* parasites and *Anopheles stephensi* mosquitos. This test can identify all known tissue schizonticidal agents and has identified and ranked according to activity several hundred compounds.

Active compounds emerging from the MM test system are selectively tested in a special secondary test system (Ag test). A wide variety of tests are performed in this test system. One aspect of this system is to identify cross resistance patterns of new active compounds against established antimalarial drugs. Many *P. falciparum* parasites in various geographic areas of the world do not respond to certain standard antimalarial agents while some of these parasites do not respond to any antimalarial agent (multiple-drug resistance). The different categories of drug resistance found in *P. falciparum* are summarized below;

1. Resistance to 4-aminoquinolines
   chloroquine

2. Resistance to arylaminoalcohols
   mefloquine (a quinolinemethanol)
   halofantrine (a phenanthrenemethanol)

3. Resistance to cinchona alkaloids
   quinine

4. Resistance to antifol drugs
   pyrimethamine
   proguanil
   Fansidar^{R}
   Fansimef^{R}

5. Resistance to acridines
   atebrine

6. Multiple-drug resistance (resistance to two or more of the above compounds.

Collectively, the several types of resistance, impair the effectiveness of all the major available antimalarials. Hence, a tremendous need exists for alternate drugs active against the various type of drug-resistant parasites.

We have lines of parasites resistant to these six categories of drug resistant types and use them to find an active compound exhibiting no cross resistance with any standard antimalarial in a multiple dose (6 day test) system.

Another approach to antimalarial chemotherapy is by using combinations of synergistically active compounds such as Fansidar^{R} (pyrimethamine plus sulfadoxine), or the triple combination of mefloquine, pyrimethamine, and sulfadoxine (Fansimef^{R}). Unfortunately both of these combinatorial drug regimens share toxicity problems due to the sulfadoxine component. New synergistically active drug combinations are needed. We have a model system to identify synergistic interactions of compounds against malaria.

The rate at which drug resistance is attained to a specific compound is very important. Resistance to pyrimethamine has been achieved within two passages whereas, resistance to chloroquine takes more than 20 passages. We have developed a standardized procedure to measure the time required to induce resistance to a specific compound. This has been used for single compounds and also for multiple drug combinations.

Several studies involved single or multiple dose schedules for selected compounds in a modified MM test system. The compounds studied were primarily artemisinin analogs.

The antioxidant status of the hosts blood system is an important component often influencing the degree of parasitemia and the eventual pathology caused by malarial organisms. These natural antioxidants may also antagonize the antimalarial activity of drugs which act via free radical formation as their primary mechanism in killing malarial parasites. Such drugs acting through the generation of free radicals include peroxides, primaquine, and qinghaosu type compounds.

By manipulating the hosts major antioxidative components in the blood (vitamin C and E) in concert with metabolically shifting the fatty acid profile of red blood cell and parasite membranes to an increased level of polyunsaturated (Pufa) omega-3 (n-3) fatty acids, one could render the infected red blood cells and the parasite more susceptible to killing by free radical acting drugs. Several studies attempting to study this three way attack on the parasite (lower antioxidant status while increasing Pufa n-3 levels in red blood cells then treating with free radical acting drug) were undertaken with very successful results.

According to the World Health Organization, there is no adequate information on the prevalence of human African trypanosomiasis. The best estimates report that 35 million people are exposed to the risk, and about 9,000 new cases are reported annually. African trypanosomiasis has a very high mortality rate and has considerable importance as a public health problem, especially in this age of increasing foreign travel.

No new antitrypanosomicidal drugs have been introduced during the past 32 years. Four drugs are currently available in the treatment of human trypanosomiasis caused by *Trypanosoma rhodesiense* or *Trypanosoma gambiense*. Three of these drugs, suramin, nitrofurazone and pentamidine are used in the treatment of the blood parasite, but lack efficacy in the treatment of central nervous system infections. The fourth drug, melarsoprol, is used in the treatment of central nervous system infections.

While these drugs are at times effective, they all have disadvantages. Suramin may cause renal damage, exfoliative dermatitis and has been shown to be teratogenic in rats. Nitrofurazone is toxic to the central nervous system and causes hemolytic anemia in glucose 6-phosphate deficient patients. Pentamidine may cause fatal hypertension, hypoglycemia and diabetes. Administration of melarsoprol leads to lethal encephalopathy in 10 to 15 percent of cases.

New compounds active against the drug susceptible trypanosomes are needed. We have developed a murine model (RR test) using *T. rhodesiense* which is reliable and susceptible to the standard drugs used today. Active compounds emerging from this primary RR test system are then tested for cross resistance patterns to each of three drug-resistant lines (a melarsoprol, a suramin, and a pentamidine-resistant line).

Infections by the hemoflagellate *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, present a devastating public health problem for millions of people in Central and South America. Reduviid bugs transmit *T. cruzi* to man via the insect's infected feces causing a disease characterized by an acute phase and a subsequent chronic degenerative phase. In addition to the cultural, social and economic factors that make Chagas' disease particularly difficult to manage, the problem is compounded by the protozoan's cellular invasiveness and its pleomorphic morphological and biochemical nature. No satisfactory course of drug therapy has been found that will treat all stages of the infection while remaining non-toxic to humans. Lampit is the primary drug to use, however, it is only active in the acute stage and has serious toxicity problems. We developed a murine test system to detect compounds active against the trypomastigote and amastigote stages of Chagas' disease. All standard compounds are active in this model system.

METHODS

MM TEST

ANIMALS HOSTS

The total supply of animals needed to screen candidate compounds was obtained from our breeding colonies of outbred ICR/HA mice until December 1985, then we used CD-1 Swiss mice (*Mus musculus*). Test animals weighed 18-20 grams. Weight variations in any given experimental or control group were carefully limited to within 2 to 3 grams. In any given test all animals were approximately the same age.

Animals on test were housed in metal-topped plastic cages, fed a standard laboratory diet and given water *ad libitum*. Once the

infected mice had been administered the drug, they were placed in a room maintained at 28.8C(±2°C), with a relative humidity of approximately 66%.

TEST PROCEDURE

Test animals received an intraperitoneal (IP) injection of approximately $6X10^5$ parasitized erythrocytes drawn from donor mice infected 4 days earlier with *Plasmodium berghei*. The donor strain was maintained by passage every 4 days in separate groups of mice inoculated with 0.2 cc of a 1:435 dilution of heparinized heart blood.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered subcutaneously (SC). Compounds to be administered orally (PO) were mixed in an aqueous solution of 0.5 hydroxyethylcellulose-0.1% Tween-80 (HEC).

Treatment consisted of a single dose given SC or PO 3 days postinfection. At the time of treatment a 10-15% parasitemia had developed. Although the disease was well established, it had not yet caused sufficient debility to affect an evaluation of the test compound's toxicity.

Deaths that occurred before the sixth day, when untreated infected controls began to die, were regarded as the result of a compound's toxic effect and not as the result of action by the infecting parasite.

Each compound was initially administered in 3 graded doses, diluted 4-fold, to groups of 5 mice per dose level. The top dose was 640, 320 or 160 mg/kg of body weight depending upon the amount of compound available for testing. Active compounds were subsequently tested at 6 or 9 dose levels, diluted 2-fold from the highest dose. Successive 6-level tests were performed at respectively lower does until the lower limit of activity was reached, thus establishing a complete dose-response picture for that compound in a rodent system.

A drug that was toxic for the host at each of the 3 levels initially tested was retested at 6 dose levels diluted 2-fold from the lowest toxic dose.

DRUG ACTIVITY

Acceptance of a drug being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. A MTD is defined as the highest dose up to 640 mg/kg causing no more than 1 of 5 animals to die from drug toxicity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the life span of untreated controls.

Clearly inactive compounds were rejected after one test, borderline compounds were characterized by a dose-response curve which established the spread the MTD and the lower limit of activity by determination of drug activity in the dose-level dilution test.

RP TEST

ANIMALS HOSTS

Male or female outbred ICR/HA Swiss mice (*Mus musculus*), 6 to 7 weeks old, weighing 16 to 17 grams were used as test animals. They were maintained in groups of 5 and had free access to feed and water.

Mice used as a source of gametocytes (donor mice) were 8 weeks old and weighted 25 to 30 grams.

MOSQUITO COLONY

Anopheles stephensi were reared in an insectary maintained at 27.7°C(±2°C) and 70% (±2%) relatively humidity with 14 hours of light and 10 hours of darkness. Larvae were fed a solution of 2.5% liver power once a day. Emerged adults were fed a 10% glucose solution *ad lib*.

INFECTED MICE AS SOURCE OF GAMETOCYTES

Donor mice to be used as a source of gametocytes were injected IP with a dilution of infected heart blood from mice previously infected with sporozoites of *Plasmodium yoelii*. These mice were used 2

to 3 days after inoculation with parasitized red blood cells. The gametocytes developed within 48 to 72 hours and produced a uniform infection in the mosquitoes.

INFECTION OF MOSQUITOES

Mosquitoes were placed in a room maintained at 21.1°C(±2°C) and 70% (±2%) relative humidity prior to receiving the infected blood meal. Donor mice harboring a 2 to 10% parasitemia were anesthetized with Nembutal and placed on top of the mosquito cages for 1 hour to allow the mosquitoes to feed on infected blood. A second infected blood meal was given the following day, and thereafter the mosquitoes were maintained on a 10% glucose solution. A single normal blood meal was given 7 days after the first infected blood meal.

ISOLATION OF SPOROZOITES

Eighteen days after the first infected blood meal the mosquitoes were anesthetized with ether, vacuumed into plastic bags kept immobilized on a cold table -5° to 0°C. The females were separated from the males and placed into a cold glass mortar. The males were discarded. After approximately 500 females were collected, one ml each of saline and heat inactivated mouse plasma were added and the suspension was macerated with a glass pestle for 3 minutes. An additional 20 ml of saline and mouse plasma (1:1) were added to the suspension and filtered through nylon monofilament screening fabric with a mesh opening of 90 microns. This step removed large tissue fragments of the mosquitoes, yet allowed the sporozoites to freely pass through into the refined suspension. This filtered sporozoite suspension was further diluted to obtain a concentration of approximately $2.5X10^5$ sporozoites per 0.2 ml. of inoculum.

ADMINISTRATION OF TEST COMPOUNDS

Each compound was ground with a mortar and pestle and then suspended in a quantity of HEC to obtain the desired drug dose. The percent free base of each compound was not determined. Four hours prior the inoculation of sporozoites, compounds were administered either SC or PO at 3 graded doses diluted four-fold (160, 40 and 10 mg/kg). Groups of 5 mice per dose level were used. Subsequent tests used successively lower four-fold dilutions of

test compound if mice were cured at 10 mg/kg until the lower limit of a compound's activity was reached.

Infected control mice (receiving sporozoites only) began to die due to malaria starting 7 days after inoculation of sporozoites. Deaths that occurred prior to 7 days in mice treated with test compounds were considered drug toxicity deaths. A drug that was toxic to the host at each of the 3 initial dose levels was retested at doses diluted four-fold from 10 mg/kg.

INOCULATION OF MICE WITH SPOROZOITES

Mice were injected IP with approximately $2.5X10^5$ sporozoites. Twenty of these mice were divided into 2 groups of 10 each. One group received no drug and served as a negative control. The other group was treated with WR 181023 (125 mg/kg) and acted as a positive control. One additional control group of 5 infected mice was treated with chloroquine (100 mg/kg).

DETERMINATION OF ANTIMALARIAL ACTIVITY

After the mice were inoculated with sporozoites they were placed in a room maintained at 28.8°C(±2C) and 66% (±2%) relative humidity. Antimalarial activity was determined by monitoring daily mortality. Mice which were alive after 30 days were considered cured. A compound was considered active if at least 2 mice survived for 30 days at any dose level. Active compounds were retested at 160, 40, 10 both SC and PO.

AG TEST

ANIMALS HOSTS

The testing was done in both female and male ICR/HA mice until December 1985 then in CD-1 mice. All mice were obtained from our own breeding colony. Four week old mice were used for most experiments except 3 week-old weanling male mice were used to start the antioxidant studies.

REGULAR 6-DAY TEST AND CROSS RESISTANCE DETERMINATIONS

When a new compound was obtained it was subjected to a battery of testing procedures, the extent of which depended on its degree

of activity in suppressing murine malaria infections. The first test procedure was a 6-day suppressive test against the drug-sensitive P-line.

If the compound was active against the P-line, then a 6-day test against one or more drug-resistant lines followed. In this basic 6-day suppressive test, mice were divided into groups of 7 and inoculated with parasites IP. Drugs were administered twice a day, usually PO, in a volume of 10 ml/kg on the third, fourth and fifth days after inoculation of parasites. All drugs were mixed in aqueous HEC and untrasonicated when necessary. Drug doses were prepared using 100% of the free base of each drug. One group of 10 infected mice received the vehicle alone and served as a negative control. The blood films and final group weights were taken on the sixth day after inoculation of parasites. Microscope examination of Giemsa-stained blood smears was made to determine the percentage of cells parasitized, percent suppression of parasitemias, and significance values for the suppression of parasitemias. Significance values were based on a calculation of the percent suppression of parasitemia which was determined by comparing the parasitemia of each treated mouse with the means parasitemia of the negative controls. Drugs tolerance was reflected by the percent weight change an the proportion of mice that survived treatment. Toxicity was attributed to drug action when a 14% or greater weight change occurred or when one or more mice died before the blood smears were taken.

Each new drug was first tested against the drug-sensitive P-line usually via both PO and SC routes of administration. The drug dosages for the first test were normally 64, 16, 4 and 1 mg/kg/day for 3 days. If more than 90% suppression of the parasitemia (SD_{90}) was obtained with the lower dose of 1 mg/kg/day, then testing at lower doses was performed. Chloroquine was tested as a reference against the P-line at levels of 2, 3, and 4 mg/kg/day. A quinine index (Q) was calculated by comparing the SD_{90} values obtained from the chloroquine dose-response curve and the SD_{90} value of the new compound:

$$Q = \frac{SD_{90} \text{ of chloroquine } 30^{\cdot}}{SD_{90} \text{ of new compound}}$$

$^{\cdot}$ = This is the quinex index for chloroquine

Compounds that suppressed the P-line parasitemia by at least 90% with 64 mg/kg or less were subjected to testing against 1 or more of the drug-resistant lines listed below;

P. berghei KBG-173

mefloquine resistant	A-line
chloroquine resistant	C-line
pyrimethamine resistant	M-line
quinine resistant	Q-line
dapsone resistant	S-line
cycloguanil resistant	T-line

P. yoelii 17X

qinghaosu resistant	U-line

P. vinckei

chloroquine resistant

Doses required for a given degree of effect, such as 90% suppression or SD_{90}'s were estimated graphically from plots made on log-probit paper. The ratio of the SD_{90}'s was used to delineate the degree of cross resistance.

SYNERGISTIC TESTS

Mice were infected IP with $5X10^4$ parasitized erythrocytes of a drug-sensitive line. The drugs were mixed separately then administered either alone or as a mixture PO twice a day on days 3, 4, and 5 after the mice had been infected. The effects were determined from blood smears made 1 day after completion of treatment. The dose suppressing 90% of the parasites (SD_{90}) for 1 drug alone and of the mixture were estimated by plotting parasitemia suppressions on probit-log scale graphs. The analyses for synergism were based upon partitioning of the SD_{90} value of each combination in terms of its components. These components were then compared with the respective SD_{90} values of the corresponding drug alone. If the joint effects were simply additive, each component of a mixture SD_{90} would be expected to be 0.5. If all values were lower than 0.5 the data would indicated synergism. Conversely, if all values were greater than 0.5 the data would indicated antagonism.

INDUCTION OF DRUG RESISTANCE

FLOXACRINE ANALOG RACEMATE AND ITS R - AND S- STEREOISOMERS

A comparative study was done to determine how fast resistance could be attained to each of the following compounds;

1.	BL 21100	A floxacrine analog racemate,
2.	BL 34170	The R-Stereoisomer of BL 21100,
3.	BL 29759	The S-Stereoisomer of BL 21100.

The drug-sensitive line of *P. yoelii* was used to start the induction of resistance. Each of 3 compounds was first administered at the same 7 dose levels (2, 1, 0.85, 0.5, 0.38, 0.25, 0.125 mg/kg/day) for 3 consecutive days b.i.d. commencing on D+3 after infection with $5X10^4$ parasitized erythrocytes. Blood films were made on D+7 and the mouse at the highest dosage level with a parasitemia of 1-5% was used as a donor mouse. This procedure was repeated weekly using the dose level passed to be the second lowest dose (X) and then the drug was increased for the next pass according the following increments.

8X
4X
3X
2X
1.5X
0.5X
0 - No drug

By using this schedule a standardized procedure was used to assess the speed that resistance to each compound was attained.

QINGHAOSU

Starting with a drug-sensitive line of *P. yoelii* qinghaosu was administered at increased levels similar to the increments stated above each week until resistance to 256 mg/kg/day was achieved.

MEFLOQUINE

Starting with the drug-sensitive P-line of *P. berghei* mefloquine was given to groups of mice at the standardized increments until resistance was attained.

SINGLE OR MULTIPLE DOSE MODIFIED MM TEST

A series of artemisinin analogs were administered either once on D+3 or multiple times on days 3, 4, and 5 after inoculation with a regular MM parasite inoculum of $6X10^5$ erythrocytes parasitized with *P. berghei*. In one test mefloquine was administered at low levels at the same time as Na artelinate to see if it would enhance its activity. In another test mefloquine was administered on days 6, 7, and 8 after Na artelinate was given on days 3, 4, and 5 postinfection.

Blood films were taken weekly starting on the sixth day after infection in all tests and continued for a 60 day period. Mice surviving the 60 day challenge were considered cured. Several standard antimalarial compounds were suspended in fat or water soluble solutions and administered once on D+3. In one test mice were treated once on D+3 and after 60 days half the mice were rechallenged while the other half were bled and their blood subinoculated into recipient mice to detect any latent parasites.

ANTIOXIDANT STUDIES

A series of 34 experiments were done in a attempt to study the influence of fatty acids and antioxidants with and without drugs on the growth of malaria. The first special study was done to determine whether a sesquiterpene endoperoxide compounds qinghaosu would exhibit enhanced antimalarial activity in mice fed a corn oil diet deficient in vitamin E. Weanling female mice were fed 1 of 3 diets; **1)** Torula-based diet without vitamin E, **2)** Torula-based diet with vitamin E, or **3)** regular mouse chow. The vitamin E consisted of all-rac-α tocopherol acetate. After 9 weeks the plasma α-tocopherol levels were determined. At this time, all mice were inoculated intraperitoneally with *P. yoelii*. Then groups of 7 to 9 mice maintained on their respective diets were given 0, 4, 16, or 64 mg/kg qinghaosu PO b.i.d. at 3, 4, and 5 days postinfection. On the 6th day, blood films were made and suppressive antimalarial activity was assessed by determining the percent parasitemia.

Survival times were monitored for 60 days postinfection. The next 3 experiments were performed to study the effect of corn oil, lard, and cod-liver oil diets deficient in vitamin E on the antimalarial activity of qinghaosu.

A series of experiments were performed to study the influence of altering the fatty acid profiles of host red blood cells and parasites membranes in infected mice fed vitamin E-deficient diets. Various polyunsaturated fatty acids high in n-3 fatty acids were used as the dietary fat source in these diets. Suppressive and curative antimalarial activity were determined by monitoring parasitemia levels and mortality data. Several experiments also were designed to study the influence of paraaminobenzoic acid (PABA) and other antioxidants on the growth of malarial parasites.

RR TEST

ANIMALS HOSTS

ICR/HA outbred mice were used until December 1985, then CD-1 Swiss mice were used. In this screening procedure mice weighed 25 to 28 grams with weight variation in any given experimental or control group carefully limited to 3 grams. Male and female mice approximately the same age were used.

Animals were housed in metal-topped plastic cages, fed a standard laboratory diet and given water *ad libitum*. After drug treatment, mice were kept in a room maintained at a temperature of 28.8°C(±2°C) with a relative humidity of 66% (±2%).

INOCULATION OF PARASITES

Test animals received and IP injection of 0.2 cc of a $1.5X10^4$ dilution of heparinized heart blood drawn from a donor mouse infected 3 days earlier (approximately $1.3X10^4$-$1.7X10^4$ trypomastigotes).

The donor line was maintained by 3-day blood passes; each animal received 0.1 cc of a $1:1.5X10^4$ dilution of heparinized heart blood drawn from a mouse harboring a 3-day infection.

One group of infected, untreated mice was included as a negative control, to check both the infectivity of the *T. rhodesiense* (CT-Wellcome strain) and the susceptibility of the murine host. In

order to determine the effect a drug exerted on a trypanosome infection, 2 parameters were measured; **1)** the increase in mouse survival time and **2)** drug curative action. For comparative purposes, 2 standard antitrypanosomal compounds, stilbamidine isethionate and 2-hydroxystilbamine isethionate, were administered SC at one dose (26.5 mg/kg) to separate groups of 10 mice each. The same positive controls were administered PO at 53 mg/kg when compounds were tested orally. These 2 diamidines served as positive controls, producing definite increase in survival time and curative effects.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered SC. Compounds to be administered PO were mixed in an aqueous solution of HEC.

Treatment consisted of a single dose, given SC or PO, 2 to 3 hours after the injection of parasites. Deaths that occurred before the 4th day, when untreated infected controls began to die, were regarded as a result of toxic action by the drug, not the lethal effects of the parasites.

Each compound was initially administered in 3 graded doses diluted 4-fold to groups of 5 mice per dose level. The top dose was either 424, 212, or 106 mg/kg, depending on the amount of compound available for testing. Active compounds were subsequently tested at 6 dose levels, diluted 2-fold from the highest dose. If necessary, successive 6-level tests were performed at respectively lower doses until the lower limit of activity was reached.

A drug that was toxic for the host at each of the 3 levels initially tested was retested at 6 dose levels diluted 2-fold from the lowest toxic dose.

DRUG ACTIVITY

Acceptance of a drug as being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. A MTD is defined as the highest dose up to 424 mg/kg causing no more than 1 of 5 animals to die from drug activity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the live span of untreated infected controls.

Clearly inactive compounds were rejected after 1 test and border-line compounds after 2 tests. Active compounds were characterized by dose-response curves, which established the spread between the MTD and the lower limit of activity by a determination of drug activity in the dose-level dilution tests. Treated animals alive at the end of 30 days were considered cured.

DRUG RESISTANT AFRICAN TRYPANOSOME TEST

ANIMAL HOSTS

SAME AS REGULAR RR TEST

INOCULATION OF PARASITES

Giemsa-stained blood smears from donor mice infected 3 days earlier with *T. rhodesiense* trypomastigotes were microscopically examined to determine parasitemias (i.e., number of trypomastigotes in a field of 100 erythrocytes). One set of test animals was infected with the drug-sensitive line of parasites by receiving an IP injection of 0.2 cc of a $1:1.5X10^4$ dilution of heparinized heart blood drawn from a donor mouse harboring a parasitemia of 30-35% (approx. $1.3X10^4$ - $1.7X10^4$ trypomastigotes). Other sets of mice were similarly infected with each drug-resistant line to be tested. Blood dilutions were made such that all mice infected with the resistant lines received approximately the same number of trypomastigotes as mice infected with the drug-sensitive line.

Groups of 10 mice per group infected with the drug-sensitive line and with each drug-resistant line but receiving no drug served as negative controls.

DRUG ADMINISTRATION

Test compounds were dissolved or suspended in either peanut oil for SC administration or HEC for PO administration. Compounds were given 1 hour following challenge with trypomastigotes.

Compounds were diluted 2 or 4-fold from a level that had been projected to be fully curative. Five mice were used for each dose level.

CROSS RESISTANCE DETERMINATION

Each compound was tested against the drug-sensitive line and the 3 drug-resistant lines (melarsoprol, suramin or pentamidine resistant). Mice surviving 30 days postinfection were considered cured. The degree of cross resistance (fold resistant) was obtained by the following calculation.

$$\text{Cross-resistance (Fold resistant)} = \frac{CD_{50} \text{ Drug-resistant line}}{CD_{50} \text{ Drug-sensitive line}}$$

CD_{50} is the lowest mg/kg level of a compound curing at least 3 of 5 mice.

CHAGAS' TEST

ANIMALS HOST

Male ICR/HA Swiss randomly bred mice 6 to 7 weeks of age weighing 18-22 grams were used.

TEST PROCEDURE

On day zero mice were inoculated IP with approximately $1.3X10^5$ trypomastigotes in blood drawn from donor mice infected 1 week previously with *T. cruzi* trypomastigotes (Y-strain). Within 30 minutes following challenge, mice were given a single SC injection of the test compound mixed in peanut oil. Each compound was initially tested at 3 dose levels, usually 640, 160, and 40 mg/kg. The end point in activity of each active compound was determined. Mortality was recorded daily for a period of 40 days after the challenge with parasites. Blood smears were taken on day 40 to detect any trypomastigotes.

Infected negative controls received an injection of the vehicle alone. This groups consistently died within 9-15 days after the IP inoculation of parasites. A positive control drug was included in each experiment. The nitrofuran, Lampit, known to have limited therapeutic value in treating patient with Chagas' disease, was used as a positive control.

A classification system was used to assess the relative activity of prospective compounds by comparing the life span of treated animals to the longevity of negative controls. Schizotrypanocidal activity was divided into 3 categories; positive, minimal and negative. A positive compound was one producing at least 50% increase in life span of mice over that of controls. A minimal compound produced a 20 to 49.9% increase in longevity, and a compound producing less than a 20% increase in life span was considered negative.

Active compounds prevented or delayed acute mortality. The test system as designed could not assure that mice living past the 40 day observation period were cured; if complete elimination of the parasite was not attained during the acute stage of infection and the animal survived, a chronic stage followed.

RESULTS AND DISCUSSION

MM TEST

In this primary mouse malaria test we tested 20,500 compounds for asexual erythrocytic activity. At least 2,100 of these compounds exhibited antimalarial activity. Therefore, approximately 10% of the compounds evaluated had at least suppressive activity with far fewer producing curative activity. The identity of most of these active compounds is considered discreet so I can not report on their chemical structures. Some compounds were of non discreet nature, and these were primarily analogs of known chemical types such as artemisinin.

RP TEST

For this sporozoite induced causal prophylactic test over 6,000 compounds were evaluated with approximately 380 exhibiting activity. This test ended in 1984. The majority of these compounds were discreet. There were false positive results obtained for compounds having no tissue schizonticidal activity but had schizonticidal activity. These false positive compounds were not sorted out from the true causal prophylactic ones.

AG TESTS

REGULAR 6-DAY TEST AND CROSS RESISTANCE DETERMINATIONS

Over 2,000 compounds were tested in special secondary antimalarial tests. Most were tested in special test using the regular 6-day test. Others were tested in the tests described below.

Mefloquine and halofantrine were tested against the chloroquine-resistant line of *P. berghei* and their suppressive effects were reduced. This indicated cross resistance with chloroquine-resistant parasites. This resistant line is a good predictor of whether a new compound will be active against chloroquine-resistant parasites since both mefloquine and halofantrine are being found to be less effective against chloroquine-resistant *P. falciparum* in nature.

The R- and S- stereoisomers of a floxacrine analog were found to interact synergistically in suppressing and curing malarial infections of *P. yoelii*. Synergistic curative activity was observed between Na artelinate and mefloquine. Four different compounds (verapamil, nifedipine, diltiazen and desipramine) did not act synergistically with chloroquine against chloroquine-resistant parasites. These tests were done in an attempt to reverse chloroquine resistance.

Inducing resistance to the 2 stereoisomers of a floxacrine analog racemate produced interesting results.

Resistance increased weekly with each compound through the fourth pass with resistance to the R-stereoisomer developing at a slower rate for the first 3 passages than the racemate or S-stereoisomers. However, by the fourth pass resistance was high for each compound. Parasites were present at 96 mg/kg level for the racemate, 64 mg/kg for the R-stereoisomer and 128 mg/kg for the S-stereoisomer. One week later when the fifth pass was to take place there were no parasites in any of the mice under drug pressure at levels of 128, 96, or 64 mg/kg. Therefore, parasites from infected non-treated control mice were passed to restart the 3 resistant lines. Resistance reappeared by the sixth pass with the S-stereoisomer, however, resistance was slower to obtain with the racemate and R-stereoisomer. Resistance to the racemate increased gradually until the seventeenth pass then parasites resistant to 64 mg/kg were obtained. The R-stereoisomer was more difficult to reinduce resistance and it wasn't until the twenty-fourth pass when

resistance was noted once again. Resistance to the R-stereoisomer of the floxacrine analogue racemate developed slower than the S-stereoisomer or the parent racemate compound. When resistance waned at the fifth passage it was more difficult to reinduce resistance to the R-stereoisomer than the S-stereoisomer or racemate.

Resistance to qinghaosu increased weekly until a top dose of 256 mg/kg/day was tolerated. This took approximately 6 weeks.

Resistance to 256 mg/kg/day of mefloquine was attained in 4 passages.

In a 3-dose modified MM test artelinic acid exhibited superior suppressive and curative antimalarial activity compared with artemisinin, Na artesunate, arteether, BL 49135, BL 49144, and BL 49153. In another test arteether suspended in sesame oil and administered once was more active SC than PO. Curative activity was obtained down to 15 mg/kg SC while 480 mg/kg was needed to cure mice orally. In a different test 2 of 3 artemisinin analogs suspended in HEC and administered PO on D+3, 4 and 5 exhibited curative activity. BL 55811 cured mice at 80 mg/kg. BL 55802 was not curative at a top dose of 320 mg/kg. Sodium artelinate administered once in HEC PO on D+3 exhibited suppressive activity at 80 mg/kg with no cures at a top dose of 160 mg/kg. When mefloquine was administered at the same time as Na artelinate an increase in suppressive and curative activity was observed at certain drug levels. Two trioxanes mixed with tyloxapol (a surfactant) then suspended in 2% methyl cellulose and administered once SC and PO on D+3 were active. BL 52276 was suppressive at 160 mg/kg SC and 640 mg/kg PO while BL 52285 was suppressive at 640 mg/kg SC but not PO at this level. Both the suppressive and curative antimalarial activity of Na artelinate and chloroquine were enhanced by a follow up 3 day course of treatment with mefloquine.

In a series of studies involving the antioxidant status of the host with and without drugs produced some dramatic results described below.

In the 1st test mice maintained on a vitamin E deficient diet containing 5% corn oil for 9 weeks did exhibit a potentiated antimalarial response to qinghaosu as determined by increased percent suppression of parasitemia values and increased survival times.

Mice given 0 or 64 mg/kg qinghaosu had average parasitemia of 25% and <1%, respectively, and this was not affected by diet. Vitamin E-deficient mice given 4 or 16 mg/kg qinghaosu had average parasitemia values of half that of the vitamin E-supplemented or chow fed mice. Survival of the mice 60 days postinfection in the groups given 16 mg/kg was 88%, 0% and 13% in the vitamin E-deficient, vitamin E-supplemented, and chow-fed groups, respectively. Vitamin E deficiency thus improved the therapeutic efficacy of qinghaosu. In a similar experiment selenium was deleted from the diet (to reduce the glutathione peroxidase enzyme system) instead of vitamin E and when qinghaosu was given no potentiation of this antimalarial activity was found.

In the second experiment 2 vitamin E-deficient diets, one with corn oil and the other with cod-liver oil, were tested for their ability to enhance the activity of qinghaosu. These diets were prefed to mice for 4 weeks before infection with *P. yoelii*. The mice fed the vitamin E-deficient cod-liver oil diet exhibit suppressed parasitemias and enhanced curative activity regardless of the level of qinghaosu.

A fourth experiment was performed to compare the activity of qinghaosu in mice fed 2 vitamin E-deficient diets containing either 5% lard or 5% corn oil. The parasites were suppressed to similar levels in mice receiving both vitamin E-deficient diets. There was an increased curative effect in the lard group receiving 8 mg/kg qinghaosu. Diphenylparaphenyldiamine (DPPD) and tert-butylhydroquinone (TBHQ) are 2 antioxidants added to the mouse diets which prevented the diets from becoming rancid but did not interfere with the development of suppression of malaria in the vitamin E-deficient diets. Rapeseed oil (canola) contains 10% n-3 fatty acids while linseed oil has a 53% level. Only linseed oil without vitamin E cured the mice. Both drug-sensitive and chloroquine-resistant parasites can be suppressed and eliminated by a vitamin E-deficient menhaden oil diet. The addition of 1% corn oil, as a source of essential fatty acids (n-6 group), to a 4% menhaden oil diet did not interfere with the curative activity obtained with vitamin E-deficient diet. Depletion of selenium did not interfere with the growth of chloroquine-resistant malaria while a depletion of vitamin E did. Twenty mg/kg of PABA added to a vitamin E supplemented menhaden oil diet allowed 50% of the parasites to grow. The 1% corn oil (containing no n-3 fatty acids) added to the 4% linseed oil (containing 53% n-3 fatty acids) when used as a fat source in a vitamin E-deficient diet did not alter its antimalarial activity. Too much TBHQ (50X) interfered with the antimalarial effect of the diets while those containing 1X or 10X

did not alter the antimalarial activity. Anchovy oil, containing similar levels of n-3 fatty acids as menhaden oil suppressed the malaria growth in a vitamin E-deficient diet comparable to that obtained with menhaden oil. Borage oil contains gamma linolenic fatty acid (an n-6 form) which suppressed the parasitemia for 1 week but produced no cures when used as the fat source in a vitamin E-deficient diet. An ethyl ester concentrate of fish oil containing 68% total eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) did not exert an increase in antimalarial activity when compared with regular menhaden oil which contains 20% EPA + DHA. A 20 mg/kg level of PABA included in a menhaden oil diet was enough to allow the malaria to grow and not be a limiting factor in the course of this experiment. A menhaden oil diet deficient in both vitamin E and PABA cured mice even when started 3 days postinfection with *P. yoelii*. Salmon oil in a vitamin E-deficient diet cured mice to the same degree as menhaden oil. Flaxseed oil cured about half the mice in a vitamin E-deficient diet. A linseed oil diet deficient in both vitamin E and PABA cured mice even when started 3 days postinfection with *P. yoelii*. Mice infected with qinghaosu-resistant parasites were cured when fed a menhaden oil diet deficient in vitamin E. The dapsone-resistant parasites grew normally in the absence of PABA but not when vitamin E levels were reduced. Three highly saturated fatty acid oils (coconut, palm kernel, and red palm) did not exert an antimalarial effect in mice depleted of vitamin E. A n-3 free fatty acid concentrate exhibited the best antimalarial activity of any polyunsaturated fatty acid type used in mice depleted of vitamin E. Suppressive and curative activity was also found in mice not depleted of vitamin E. Qinghaosu-resistant and chloroquine-resistant parasites did not grow when vitamin E and PABA were depleted in the mice. Parasites of *P. yoelii* developed and killed mice when a range of 8.75 to 70 mg/kg of vitamin E was administered. The polyunsaturated fatty acids from *Spirulina* algae did not exert an antimalarial effect in mice depleted of vitamin E. Twice the normal level of polyunsaturated fatty acids did not increase the antimalarial activity in mice fed this diet beginning on the 3rd day after infection. Curative activity was observed in PABA deficient groups receiving vitamin E even when the diet was started 3 days postinfection. Four fatty acids (stearic acid, linoleic, oleic acid and elapid acid) did not alter the growth of *P. yoelii* in mice depleted of vitamin E. Linseed meal exerted marked antimalarial activity when fed to mice depleted of vitamin E. Feeding the ethyl ester of the easily peroxidized highly polyunsaturated fatty acid, linolenic acid, to vitamin E-deficient mice conferred protection against *P. yoelii*. Ascorbital palmitate did not interfere with the

antimalarial activity of the menhaden oil diet deficient in vitamin E. Both synthetic and natural alpha vitamin E reversed the suppressive antimalarial activity of a polyunsaturated fatty and diet in mice infected with *P. yoelii*. Gamma vitamin E did not interfere with the suppressive or curative antimalarial activity of mice fed the same fatty acid diet. *Plasmodium vinckei* parasite growth was not influence by a depletion of vitamin E, whereas, a deficiency in PABA retarded its growth. This was true even with a low parasite inoculum. Therefore, this parasite species differs markedly from *P. berghei*. Regarding the influence of the antioxidant vitamin E. Large doses when 2000 mg/kg of vitamin C was administered on day -1, 0, 1, and 2 postinfection to mice depleted of vitamin E, it had no influence on the development of malaria.

RR TEST

In the primary drug test system for African trypanosomes (RR test) 16,688 compounds were evaluated with 784 exhibiting antitrypomastigote activity.

DRUG RESISTANT AFRICAN TRYPANOSOME TEST

In the drug-resistant African trypanosome test system 261 compounds were tested against the 3 lines resistant to either melarsoprol, suramin or pentamidine. The structures of the antitrypanosome compounds are discreet.

CHAGAS' TEST

In the Chagas' disease test system 700 compounds were evaluated for activity with 50 exhibiting antitrypanosome activity. These compounds were also discreet.

CONCLUSIONS

The primary murine malaria test (MM) continues to identify new compounds and the best analogs from lead directed synthesis program. About 10% of the total compounds evaluated were active (2,100 of 20,500).

The prophylactic antimalarial test (RP) identified 380 compounds as active out of over 6000 tested. If a compound had prolongs blood schizonticidal activity and no tissue schizonticidal activity it could be positive in this test. These false positive results were not separated from the true casual prophylactic compounds. This test has ended but it still is useful to identify possible tissue schizonticidal activity.

The secondary antimalarial test system (Ag) continues to evaluate selected active compounds in a variety of different test types. The time required to induce resistance to a compound will vary. Resistant parasites were obtained quicker to mefloquine than qinghaosu. It took longer to induce resistance to the R-stereoisomer than the S-stereoisomer of a floxacrine racemate analog. A new compound needs to be tested against the battery of drug-resistant parasites. Very few compounds have emerged with no observable cross resistance to the standard antimalarials. Artemisinin analogs exhibiting the best antimalarial activity can be easily identified in the multiple dose modified MM test. Artelinic acid appears to be the most active analog. When the parasite and host red blood cell membrane fatty acids are changed to a more polyunsaturated level (increase in omega-3 forms) and the antioxidant status of the host is altered (primarily by a reduction of vitamin E levels) the activity of qinghaosu can be potentiated. Mice can even be cured from malaria by altering the fatty acid and vitamin E levels without drug intervention. This is true for drug-sensitive parasites and those resistant to either chloroquine, qinghaosu, or dapsone. *Plasmodium vinckei* drug-sensitive parasites grew in the absence of vitamin E in contrast to *P. berghei* and *P. yoelii* parasites whose growth is dramatically reduced.

In the primary African trypanosome test (RR) 784 compounds out of 16,688 were active against the trypomastigote stage. This test was very reliable and identified several new active classes of compounds. This test system was stopped in January 1989 so more time could be spent working on malaria.

The drug-resistant test system for the African trypanosomes evaluated 261 compounds for cross resistance against lines resistant to either melarsoprol, suramin, or pentamidine. Several compounds emerged showing no cross resistance with these 3 standard agents. This test system was stopped in January 1989 when more emphasis was placed on malaria.

The test to detect compounds active against Chagas' disease identified 50 as active out of 700 tested. This test was reliable but was stopped in 1983 also to do more work in malaria.

With malaria on the increase worldwide today new compounds need to be identified to combat this major disease.

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